



TITLE:

Metabolic engineering for the production of polyunsaturated fatty acids by oleaginous fungus *Mortierella alpina* 1S-4.

AUTHOR(S):

Sakuradani, Eiji; Ando, Akinori; Shimizu, Sakayu; Ogawa, Jun

CITATION:

Sakuradani, Eiji ...[et al]. Metabolic engineering for the production of polyunsaturated fatty acids by oleaginous fungus *Mortierella alpina* 1S-4.. *Journal of bioscience and bioengineering* 2013, 116(4): 417-422

ISSUE DATE:

2013-10

URL:

<http://hdl.handle.net/2433/178746>

RIGHT:

© 2013 The Society for Biotechnology, Japan. Published by Elsevier B.V.; This is not the published version. Please cite only the published version.; この論文は出版社版ではありません。引用の際には出版社版をご確認ご利用ください。

**Metabolic engineering for the production of polyunsaturated fatty acids by
oleaginous fungus *Mortierella alpina* 1S-4**

Running Title: Microbial production of polyunsaturated fatty acids

Eiji Sakuradani,^{1,*} Akinori Ando,² Sakayu Shimizu,³ Jun Ogawa¹

Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University,
Sakyo-ku, Kyoto 606-8502, Japan¹, Research Division of Microbial Sciences, Kyoto
University, Sakyo-ku, Kyoto 606-8502, Japan², and Faculty of Bio-environmental
Science, Kyoto Gakuen University, Kameoka, Kyoto 621-8555, Japan³

* Corresponding author, Tel: +81 (0) 75 753 6114; fax: +81 (0) 75 753 6128.

E-mail address: esakura@kais.kyoto-u.ac.jp (E. Sakuradani).

1 **Researches related with the application of functional lipids such as**
2 **polyunsaturated fatty acids (PUFAs) have been conducted in various fields with a**
3 **view to health and dietary requirements. Novel rich sources other than known**
4 **natural sources such as plant seeds and fish oils are required for increasing**
5 **demands of PUFAs. The filamentous fungus *Mortierella alpina* 1S-4 produces**
6 **triacylglycerols rich in arachidonic acid, i.e., ones reaching 20 g/l in concentration**
7 **and containing 30-70% arachidonic acid as total fatty acids. Various mutants**
8 **derived from *M. alpina* 1S-4 have led to the production of oils containing various**
9 **PUFAs. Molecular breeding of *M. alpina* strains by means of manipulation of the**
10 **genes involved in PUFA biosynthesis facilitates improvement of PUFA productivity**
11 **and elucidation of the functions of their enzymes. This review describes practical**
12 **PUFA production through mutant breeding, functional analyses of the genes of the**
13 **enzymes involved in PUFA biosynthesis, and recent advances in unique PUFA**
14 **production through molecular breeding.**

15
16 **[Key words: *Mortierella alpina*; Polyunsaturated fatty acid; Arachidonic acid (AA);**
17 **Eicosapentaenoic acid (EPA); Molecular breeding; Fatty acid desaturase]**

Polyunsaturated fatty acids (PUFAs) contain more than one double bond, and some 20-carbon (C20) PUFAs play important roles not only as structural components of membrane phospholipids but also as precursors of eicosanoids, signaling molecules including prostaglandins, thromboxanes, and leukotrienes, that are essential for all mammals. Especially, arachidonic acid (AA; 20:4n-6), a representative n-6 PUFA, is the most abundant C20 PUFA in humans, and not only exhibits various regulation effects and physiological activities but also plays important roles in infant nutrition (1,2). Eicosapentaenoic acid (EPA; 20:5n-3), a representative n-3 PUFA, is beneficial in the treatment of cardiovascular diseases (3), and decreases platelet aggregation and blood pressure (4). The distinct functions of the two families make the ratio in the diet of n-6 and n-3 PUFAs important in inflammatory responses and cardiovascular health. The most readily available lipid sources relatively rich in C20 PUFAs, none of which are found in plants, are fish oils, animal tissues, and algal cells. Transgenic plants with some exogenous desaturase genes have been reported to produce n-3 and n-6 PUFAs (5). However, these transgenic sources are unsuitable for practical purposes from the viewpoint of genetically modified organisms. The term "Single Cell Oils" is used for unique oils produced by microorganisms that compete with plant-seed oils and fish oils (6). Some yeasts and molds are known as microorganisms that accumulate high levels of triacylglycerols. A lipid content in excess of 40% (w/w) is not exceptional, and values of 70% and even 80% have been reported (7). Single Cell Oils having different fatty acid compositions from plant-seed oils and fish oils are valuable for human life.

On screening of the microorganisms accumulating C20 PUFAs, a filamentous fungus, *Mortierella alpina* 1S-4, was isolated as a suitable source for the AA production; it was able to produce EPA through the n-3 PUFA biosynthetic pathway, while AA through the

n-6 PUFA biosynthetic pathway (8-10). In this strain, most PUFAs are present in triacylglycerols as storage oils, while some are present in phospholipids as structural components of membranes.

Although success in this area over the last 25 years has generated much interest in the development of microbial fermentation processes, manipulation of the lipid compositions of microorganisms requires new biotechnological strategies to obtain high yields of the desired PUFAs. This article reviews recent progress in the breeding of commercially important arachidonic acid-producing *M. alpina* strains, particularly approaches to creating desaturase and elongase mutants with unique pathways for PUFA biosynthesis involving conventional chemical mutagenesis and modern molecular genetics.

VARIOUS KINDS OF PUFAs IN *M. alpina* 1S-4

Isolation of mutants producing PUFAs through different biosynthetic pathways

Various mutants defective in desaturase ($\Delta 9$, $\Delta 12$, $\Delta 6$, $\Delta 5$ and $\omega 3$) or elongase (MALCE1) activities, or with enhanced desaturase activities ($\Delta 6$ and $\Delta 5$) have been derived from *M. alpina* 1S-4 by treating the parental spores with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (11). In addition, a diacylglycerol-accumulating mutant and several lipid-excretive ones have been obtained by the same method. They are valuable not only as producers of useful PUFAs (novel or already existing) but also for providing valuable information on PUFA biosynthesis in this fungus (12). The main features of these mutants are summarized in Table 1.

$\Delta 9$ Desaturase-defective mutants accumulate stearic acid (18:0) as the main fatty acid

(up to 40%) in the mycelial oil (13). $\Delta 12$ Desaturase-defective mutants accumulate high levels of n-9 PUFAs, such as Mead acid (MA; 20:3n-9) that are not detected in the wild strain because of a complete deficiency of $\Delta 12$ desaturation (Fig. 1A). One of these mutants, JT-180, yields a large amount of MA (2.6 g /L, 49% in oil) on commercial production due to its enhanced $\Delta 5$ and $\Delta 6$ desaturase activities, not including n-6 and n-3 PUFAs (14). Double mutants defective in both $\Delta 12$ and $\Delta 5$ desaturase activities accumulate n-9 eicosadienoic acid (20:2n-9) as a final product of n-9 PUFAs in large quantities (15). $\Delta 6$ Desaturase-defective mutants accumulate linoleic acid (18:2n-6) as the main fatty acid (up to 32%) in the mycelial oil (16). These mutants are characterized by the accumulation of n-6 eicosadienoic acid (20:2n-6) and nonmethylene-interrupted n-6 eicosatrienoic acid (20:3n-6 $\Delta 5$) synthesized from linoleic acid, as shown in Fig. 1B. $\Delta 5$ Desaturase-defective mutants exhibit a high dihomo- γ -linolenic acid (DGLA; 20:3n-6) level (4.1 g/L, 42% in oil) and a reduced concentration (<1%) of AA (17). One of these mutants, S14, is used for the commercial production of DGLA. $\omega 3$ Desaturase-defective mutants are unable to synthesize n-3 PUFAs at temperatures below 20°C (18), although the wild strain accumulates n-3 PUFAs such as EPA below that temperature. Therefore, these $\omega 3$ -desaturase defective mutants are superior to the wild strain for lipid production with a relatively high content of AA. The fatty acid profile of elongase (EL1 for the conversion of palmitic acid, 16:0, to 18:0)-defective mutants is characterized by high levels of 16:0 and palmitoleic acid (16:1n-7), with small amounts of various kinds of n-7 and n-4 PUFAs, as shown in Fig. 1c, which are not detected in the wild strain. The total content of these PUFAs in the oil reaches about 30%. In a similar manner, n-1 PUFAs can be produced from n-1 hexadecenoic acid (16:1n-1) or 1-hexadecene added

to the medium (see Fig. 1C). Triacylglycerols produced by *M. alpina* 1S-4 account for 90% of the total lipids, whereas diacylglycerol-accumulating mutant KY1 derived from the wild strain accumulates 30% diacylglycerols in the total lipids. Lipid-excretive mutant V6 shows the same lipid productivity and fatty acid composition as the wild strain, and excretes 10-40% of the total lipids into the medium during submerged cultivation. Many lipid particles containing triacylglycerols are observed on the surface of V6 mycelia cultivated on a solid medium. V6 is assumed to excrete accumulated lipids out of its mycelia due to its insufficient cell wall structure caused by mutations in the metabolic pathways for cell wall synthesis.

Characterization of enzyme genes involved in PUFA biosynthetic pathways The genes encoding the fatty acid desaturases and elongases involved in C20 PUFA biosynthesis in *M. alpina* 1S-4 and its mutant strains were characterized, as described below. These nucleotide sequence information revealed mutation sites of the enzymes in the representative mutants as described above (see Table 1).

The three $\Delta 9$ desaturase homologues (designated as $\Delta 9$ -1, $\Delta 9$ -2, and $\omega 9$) in *M. alpina* 1S-4 has a cytochrome *b*₅-like domain linked to its carboxyl terminus, as also seen for the yeast $\Delta 9$ desaturase (19). *Mortierella* $\Delta 9$ -1 exhibits 45% and 34% amino acid sequence similarity with those of *Saccharomyces cerevisiae* and rat, suggesting that the *Mortierella* $\Delta 9$ -1 is a membrane-bound protein using acyl-CoA as substrates. Both $\Delta 9$ -1 and $\Delta 9$ -2 desaturate 18:0 to oleic acid (18:1n-9), whereas $\omega 9$ desaturates a very long saturated fatty acid (26:0) to the corresponding monounsaturated fatty acid (26:1n-9) (20). Although the $\Delta 9$ -2 gene is not transcribed in the wild strain, the $\Delta 9$ -2 gene is transcribed and its derivative enzyme exhibits $\Delta 9$ desaturation activities in $\Delta 9$

desaturation-defective mutants which have a mutation site in its $\Delta 9-1$ gene. *Mortierella* $\Delta 5$ and $\Delta 6$ desaturases have a cytochrome b_5 -like domain linked to its N-terminus. The two $\Delta 6$ desaturase homologues (designated as $\Delta 6-1$ and $\Delta 6-2$) are in the wild strain, in which the $\Delta 6-1$ gene is transcribed much more highly (2- to 17-fold) than the $\Delta 6-2$ one (21). *Mortierella* $\Delta 12$ and $\omega 3$ genes lacking a region encoding a cytochrome b_5 -like domain were cloned and characterized by means of heterologous gene expression systems. The gene expression analysis in yeast revealed that *Mortierella* $\omega 3$ desaturase converts n-6 PUFAs to n-3 PUFAs with carbon 18 and 20 chain lengths, especially *Mortierella* $\omega 3$ desaturase could effectively convert AA into EPA (22).

M. alpina 1S-4 possesses 4 kinds of genes encoding fatty acid elongases (MALCE1, MALCE2, GLELO, and MAELO) involved in long chain saturated fatty acid or PUFA biosynthetic pathways. MALCE1 and MALCE2 belong to $\Delta 9$ elongases which efficiently perform elongation of 16:1n-7, 18:2n-6, and 18:3n-3. Furthermore, MALCE1 plays an important role in the elongation of 16:0 to 18:0 in *M. alpina* 1S-4 (23). GLELO is a $\Delta 6$ elongase which plays a critical role in the elongation of both C18 n-3 and C18 n-6 PUFAs to the corresponding C20 PUFAs (24). The enzyme encoded by the *maelo* gene was demonstrated to be involved in the biosynthesis of saturated fatty acids (20:0, 22:0, and 24:0) in *M. alpina* 1S-4 (25).

GENETIC MANIPULATION OF *M. alpina* STRAINS FOR PUFA PRODUCTION

Development of a transformation system for *M. alpina* strains A transformation

system for *M. alpina* 1S-4 has been developed with *M. alpina* uracil auxotrophs and a complementary gene as selective marker (26). *M. alpina* uracil auxotrophs were isolated by spontaneous mutation on a solid medium containing 5-fluoroorotic acid, of which 0.5 mg/ml inhibited the growth of the wild strain completely, with the addition of a little uracil. The uracil auxotrophs were proved each to have a point mutation in the *ura5* gene encoding orotidine-5'-monophosphate decarboxylase. These uracil auxotrophs, used in the transformation as hosts, were confirmed to exhibit the same lipid productivity, AA productivity, growth speed, and spore formation capacity as the wild strain.

Transformation with spores of *M. alpina* 1S-4 and a vector containing the *M. alpina* 1S-4 *ura5* gene as a marker has been performed through microprojectile bombardment (27), while other methods frequently used for transformation, such as ones involving protoplasting, lithium acetate, and electroporation, did not give satisfactory results, because of the difficulty of effective protoplast formation by the use of general and commercial lytic enzymes, such as chitinase, chitosanase, and glucanase, for cell walls. Transformants were obtained at a transformation frequency of 0.4 transformants/ μ g of vector DNA. Southern blot analysis revealed that most of the integrated plasmids in the stable transformants were present as several copies at ribosomal DNA (rDNA) positions and/or random positions in the chromosomal DNA.

An *Agrobacterium tumefaciens*-mediated transformation system for *M. alpina* 1S-4 has been developed (28), in which the *ura5* gene is used as a selectable marker under the control of the homologous histone H4.1 promoter in the transfer-DNA region. The frequency of transformation reached more than 400 transformants/ 10^8 spores. Southern blot analysis revealed that most of the integrated transfer-DNA appeared as a

single copy at a random position in the chromosomal DNA.

M. alpina 1S-4 exhibits resistance to various antibiotics used in the transformation systems of filamentous fungi. A high concentration (20 mg/ml) of Zeocin completely inhibited the germination of *M. alpina* 1S-4 spores, and decreased the rate of growth of fungal filaments to some extent. *M. alpina* 1S-4 showed Zeocin resistance with integration of the Zeocin-resistance gene at the rDNA locus of the genomic DNA (29). On the other hand, the fungicide carboxin (100 µg/ml) was found to inhibit the hyphal growth and spore germination of *M. alpina* 1S-4 completely (30). The *sdhB* gene encodes the iron-sulfur (Ip) subunit of the succinate dehydrogenase (SDH, EC 1.3.99.1) complex. The mutated *sdhB* (*CBXB*) gene, which leads to an amino acid substitution (H243L, a highly conserved histidine residue within the third cysteine-rich cluster of SDHB being replaced by a leucine residue), conferred carboxin resistance. The transformants obtained with the homologous *CBXB* gene from *M. alpina* 1S-4 as a selective marker exhibited carboxin resistance. The *sdhC* gene encoding a subunit of the SDH complex was also isolated from *M. alpina* 1S-4. The *sdhC* gene has been reported to act as a selectable marker instead of the *sdhB* gene (31). In the same manner, a mutated *sdhC* (*CBXC*) gene was constructed to encode a modified SdhC with an amino acid substitution (H83K and T86I, highly conserved histidine and threonine residues within a putative SDH quinone-binding site of SDHC being replaced by lysine and isoleucine ones respectively). Transformants obtained with a *CBXC* plasmid exhibited carboxin resistance, too. These genes for Zeocin and carboxin resistance are thus useful as selective markers in the transformation not only of the parental strain, *M. alpina* 1S-4, but also of its mutants.

PUFA production through molecular breeding of *M. alpina* A practical transformation system for *M. alpina* 1S-4 allows overexpression and RNA interference (RNAi) of the genes involved in PUFA biosynthesis for improvement of the production of various PUFAs. The valuable *Mortierella* mutants derived by chemical mutagenesis were directly transformed with drug resistance markers, or their uracil auxotrophs were transformed with the *ura5* marker. Molecular breeding of *M. alpina* 1S-4 and its mutants led to unique fatty acid profiles and high productivities of valuable PUFAs, as summarized in Table 2.

Mutant JT-180 exhibits no activities of $\Delta 12$ desaturase and enhanced $\Delta 5$ and $\Delta 6$ desaturase activities. On overexpression of the endogenous $\Delta 12$ gene in JT-180, it accumulated a higher amount of AA (2.0 g/l/7 d, 39% of total fatty acids), instead of MA, due to both enhanced $\Delta 5$ and $\Delta 6$ desaturation as compared to the case of the wild strain (1.2 g/l/7 d, 21%).

Expression of the gene encoding GLELO, which has been suggested to be the limiting step in AA biosynthesis (32), was successfully performed in *M. alpina* 1S-4 (33). The resulting transformants yielded more AA (3.6 g/l/10 days, 28%) than the wild strain (1.9 g/l/10 days, 19%). In addition, overexpression of the endogenous *malce1* gene in *M. alpina* 1S-4 also led to faster and higher AA accumulation (0.76 g/l/6 d, 34%) than in the wild strain 1S-4 (0.68 g/l/6 d, 28%). Overexpression of both *malce1* and *glelo* genes had significant effects on AA production by *M. alpina* 1S-4.

The $\Delta 5$ and $\Delta 6$ (Pav $\Delta 5$ and Ost $\Delta 6$) desaturases from microalgae *Pavlova salina* and *Ostreococcus lucimarinus*, respectively, have desaturation activities for acyl-CoA forms of substrates. On the other hand, the $\Delta 5$ and $\Delta 6$ desaturases from *M. alpina* make phospholipids their substrates. By gene expression of these microalgal desaturases

with different substrate specificities for fatty acid derivatives in *M. alpina*, higher contents of PUFAs might be obtained through molecular breeding. Overexpression of the *PavΔ5* gene in the wild strain led to a high rate of AA and a quite low rate of DGLA in the total fatty acids, compared with AA and DGLA rates in the wild strain. As the same manner, overexpression of the *OstΔ6* gene in the wild strain led to a total higher rate of 18:3n-6, DGLA and AA in the total fatty acid than that in the wild strain

Overexpression of the endogenous $\omega 3$ gene in the wild strain and S14 ($\Delta 5$ desaturation-defective mutant) led to higher production of EPA (0.8 g/l, 30%) as shown in Fig. 2 and 20:4n-3 (1.8 g/l, 35%), which usually comprise about 10% of the total fatty acids in the wild strain and S14 cultivated at low temperatures (<20°C). Molecular breeding of $\omega 3$ gene-overexpressing transformants gave only high productivities of these n-3 C20 PUFAs. Overexpression of both the elongase *PavELO* (involved in the conversion of C20 to C22 PUFAs in marine microalga *Pavlova* sp.) and $\omega 3$ genes in the wild strain led to the formation of C22 PUFAs, n-6 docosatetraenoic acid (22:4n-6) and n-3 docosapentaenoic acid (22:5n-3).

RNAi method with double strand RNA was applied to silencing gene expression in *M. alpina* 1S-4 (34). $\Delta 12$ Gene-silenced strains accumulated n-9 octadecadienoic acid (18:2n-9), 20:2n-9, and MA, which are not detected in either the control strain or wild-type strain 1S-4. The fatty acid composition of these transformants was similar to that of $\Delta 12$ desaturation-defective mutants previously identified. Thus RNAi can be used to alter the types and relative amounts of fatty acids produced by commercial strains of this fungus as a simple method of silencing gene expression.

The RNAi of the $\Delta 12$ gene in MALCE1 activity-defective mutant M1 led to an accumulation of n-7 PUFAs and a decrease in n-4 PUFAs. This indicates that n-4

PUFAs are biosynthesized from n-7 PUFAs by $\Delta 12$ desaturation. In addition, the M1 transformant obtained on RNAi of the *maelo* gene accumulated n-4/n-7 PUFAs with a decrease in n-6 PUFAs, which suggests that MAELO is involved in the elongation not only of long chain saturated fatty acids such as 20:0 and 22:0 but also of 16:0. Such molecular breeding of *M. alpina* strains should facilitate improvement of PUFA productivity and elucidation of the functions of the enzymes involved in PUFA biosynthesis.

CONCLUSION

The studies described above summarize our results related to PUFA production by oleaginous fungus *M. alpina* 1S-4 and the elucidation of fungal lipogenesis involved in PUFA biosynthesis. *M. alpina* 1S-4 and derivative mutants are potential sources of triacylglycerols rich in various PUFAs, including n-1, n-3, n-4, n-6, n-7, and n-9 PUFAs. It is possible to control the fatty acid profiles of fungal mutants and to regulate the flow of glucose or exogenous fatty acids to obtain a desired PUFA. The recent study on *M. alpina* and its mutants have been focused on molecular engineering of the enzyme genes involved in PUFA biosynthesis and pioneered the improvement of PUFA productivity. The breeding of mutants and transgenic strains may make it possible to produce desired PUFAs effectively. Further development of an efficient gene expression system for unique heterologous genes involved in such processes as lipid synthesis, PUFA synthesis, and lipid conversion, and construction of a homologous gene disruption system for *M. alpina* 1S-4 will enable elaboration of metabolic engineering for the production of various lipids with industrial interests.

ACKNOWLEDGEMENTS

This work was partially supported by Grants-in Aid for Scientific Research (no. 22380051 to E.S. and no. 23248014 to J.O.), the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry, and Advanced Low Carbon Technology Research and Development Program.

References

1. **Carlson, S. E., Werkman, S. H., Peeples, J. M., Cooke, R. J., and Tolley, E. A.:** Arachidonic acid status correlates with first year growth in preterm infants, *Proc. Natl. Acad. Sci. USA*, **90**, 1073-1077 (1993).
2. **Gill, I. and Valivety, R.:** Polyunsaturated fatty acids, part 1: Occurrence, biological activities and application, *Trends Biotechnol.*, **15**, 401-409 (1997).
3. **Shimokawa, H.:** Beneficial effects of eicosapentaenoic acid on endothelial vasodilator functions in animals and humans, *World Rev. Nutr. Diet.*, **88**, 100-108 (2001).
4. **Mori, T. A., Beilin, L. J., Burke, V., Morris, J., and Ritchie, J.:** Interactions between dietary fat, fish, and fish oils and their effects on platelet function in men at risk of cardiovascular disease, *Arterioscler. Thromb. Vasc. Biol.*, **17**, 279-286 (1997).
5. **Qi, B., Fraser, T., Mugford, S., Dobson, G., Sayanova, O., Butler, J., Napier, J. A., Stobart, A. K., and Lazarus, C. M.:** Production of very long chain

- 1 polyunsaturated ω 3 and ω 6 fatty acids in plants. Nat. Biotechnol., **22**, 739-745
- 2 (2004).
- 3 6. **Ratledge, C.:** Microbial lipids: Commercial realities or academic curiosities, pp.
- 4 1-15, in: Kyle, D. J. and Ratledge, C. (Eds), Industrial applications of single cell oils,
- 5 AOCS Press, IL, (1992).
- 6 7. **Ratledge, C.:** Single cell oils — have they a biotechnological future?, Trends
- 7 Biotechnol., **11**, 278–284 (1993).
- 8 8. **Shimizu, S., Kawashima, H., Shinmen, Y., Akimoto, K., and Yamada, H.:**
- 9 Production of eicosapentaenoic acid by *Mortierella* fungi, J. Am. Oil Chem. Soc., **65**,
- 10 1455-1459 (1988).
- 11 9. **Shinmen, Y., Shimizu, S., and Yamada, H.:** Production of arachidonic acid by
- 12 *Mortierella* fungi: selection of a potent producer and optimization of culture
- 13 conditions for large-scale production, Appl. Microbiol. Biotechnol., **31**, 11-16
- 14 (1989).
- 15 10. **Yamada, H., Shimizu, S., and Shinmen, Y.:** Production of arachidonic acid by
- 16 *Mortierella elongata* 1S-5, Agric. Biol. Chem., **51**, 785-790 (1987).
- 17 11. **Jareonkitmongkol, S., Shimizu, S., and Yamada, H.:** Fatty acid desaturation
- 18 defective mutants of an arachidonic acid-producing fungus, *Mortierella alpina* 1S-4,
- 19 J. Gen. Microbiol., **138**, 997-1002 (1992).
- 20 12. **Certik, M., Sakuradani, E., and Shimizu, S.:** Desaturase-defective fungal
- 21 mutants: useful tools for the regulation and overproduction of polyunsaturated fatty
- 22 acids, Trends Biotechnol., **16**, 500-505 (1998).
- 23 13. **Jareonkitmongkol, S., Sakuradani, E., and Shimizu, S.:** Isolation and
- 24 characterization of a Δ 9-desaturation-defective mutant of an arachidonic

- 1 acid-producing fungus, *Mortierella alpina* 1S-4, J. Am. Oil Chem. Soc., **79**,
- 2 1021-1026 (2002).
- 3 14. Sakuradani, E., Kamada, N., Hirano, Y., Nishihara, M., Kawashima, H.,
- 4 Akimoto, K., Higashiyama, K., Ogawa, J., and Shimizu, S.: Production of
- 5 5,8,11-*cis*-eicosatrienoic acid by a $\Delta 5$ and $\Delta 6$ desaturation activity-enhanced mutant
- 6 derived from a $\Delta 12$ desaturation activity-defective mutant of *Mortierella alpina*
- 7 1S-4, Appl. Microbiol. Biotechnol., **60**, 281-287 (2002).
- 8 15. Kamada, N., Kawashima, H., Sakuradani, E., Akimoto, K., Ogawa, J., and
- 9 Shimizu, S.: Production of 8,11-*cis*-eicosadienoic acid by a $\Delta 5$ and $\Delta 12$
- 10 desaturase-defective mutant derived from the arachidonic acid producing fungus
- 11 *Mortierella alpina* 1S-4, J. Am. Oil Chem. Soc. **76**, 1269-1274 (1999)
- 12 16. Jareonkitmongkol, S., Shimizu, S., and Yamada, H.: Occurrence of two
- 13 nonmethylene-interrupted fatty acids in a $\Delta 6$ -desaturase-defective mutant of the
- 14 fungus *Mortierella alpina* 1S-4, Biochim. Biophys. Acta, **1167**, 137-141 (1993).
- 15 17. Jareonkitmongkol, S., Sakuradani, E., and Shimizu, S.: A novel
- 16 $\Delta 5$ -desaturase-defective mutant of *Mortierella alpina* 1S-4 and its
- 17 dihomio- γ -linolenic acid productivity, Appl. Environ. Microbiol., **59**, 4300-4304
- 18 (1993).
- 19 18. Sakuradani, E., Hirano, Y., Kamada, N., Nojiri, M., Ogawa, J., and Shimizu,
- 20 S.: Improvement of arachidonic acid production by mutants with lowered
- 21 $\omega 3$ -desaturation activity derived from *Mortierella alpina* 1S-4, Appl. Microbiol.
- 22 Biotechnol., **66**, 243-248 (2004).
- 23 19. Sakuradani, E., Kobayashi, M., and Shimizu, S.: Δ^9 -Fatty acid desaturase from
- 24 arachidonic acid-producing fungus. Unique gene sequence and its heterologous

- 1 expression in a fungus, *Aspergillus*, Eur. J. Biochem., **260**, 208-216 (1999).
- 2 20. Abe, T., Sakuradani, E., Asano, T., Kanamaru, H., and Shimizu, S.: Functional
- 3 characterization of $\Delta 9$ and $\omega 9$ desaturase genes in *Mortierella alpina* 1S-4 and its
- 4 derivative mutants, Appl. Microbiol. Biotechnol., **70**, 711-719 (2006).
- 5 21. Sakuradani, E. and Shimizu, S.: Gene cloning and functional analysis of a second
- 6 $\Delta 6$ -fatty acid desaturase from an arachidonic acid-producing *Mortierella* fungus,
- 7 Biosci. Biotechnol. Biochem., **67**, 704-711 (2003).
- 8 22. Sakuradani, E., Abe, T., Iguchi, K., and Shimizu, S.: A novel fungal
- 9 $\omega 3$ -desaturase with wide substrate specificity from arachidonic acid-producing
- 10 *Mortierella alpina* 1S-4, Appl. Microbiol. Biotechnol., **66**, 648-654 (2005).
- 11 23. Sakuradani, E., Nojiri, M., Suzuki, H., and Shimizu, S.: Identification of a novel
- 12 fatty acid elongase with a wide substrate specificity from arachidonic
- 13 acid-producing fungus *Mortierella alpina* 1S-4, Appl. Microbiol. Biotechnol., **84**,
- 14 709-716 (2009).
- 15 24. Parker-Barnes, J. M., Das, T, Bobik, E., Leonard, A. E., Thurmond, J. M.,
- 16 Chaung, L. T., Huang, Y. S., and Mukerji, P.: Identification and characterization
- 17 of an enzyme involved in the elongation of n-6 and n-3 polyunsaturated fatty acids,
- 18 Proc. Natl. Acad. Sci. USA, **97**, 8284-8289 (2000).
- 19 25. Sakuradani, E., Murata, S., Kanamaru, H., and Shimizu, S.: Functional analysis
- 20 of a fatty acid elongase from arachidonic acid-producing *Mortierella alpina* 1S-4,
- 21 Appl. Microbiol. Biotechnol., **81**, 497-503 (2008).
- 22 26. Takeno, S., Sakuradani, E., Murata, S., Inohara-Ochiai, M., Kawashima, H.,
- 23 Ashikari, T., and Shimizu, S.: Cloning and sequencing of the *ura3* and *ura5* genes,
- 24 and isolation and characterization of uracil auxotrophs of the fungus *Mortierella*

- 1 *alpina* 1S-4, Biosci. Biotechnol. Biochem., **68**, 277-285 (2004).
- 2 27. **Takeno, S., Sakuradani, E., Murata, S., Inohara-Ochiai, M., Kawashima, H.,**
- 3 **Ashikari, T., and Shimizu, S.:** Establishment of an overall transformation system
- 4 for an oil-producing filamentous fungus, *Mortierella alpina* 1S-4, Appl. Microbiol.
- 5 Biotechnol., **65**, 419-425 (2004).
- 6 28. **Ando, A., Sumida, Y., Negoro, H., Suroto, D. A., Ogawa, J., Sakuradani, E., and**
- 7 **Shimizu, S.:** Establishment of *Agrobacterium tumefaciens*-mediated transformation
- 8 of an oleaginous fungus, *Mortierella alpina* 1S-4, and its application for
- 9 eicosapentaenoic acid producer breeding, Appl. Environ. Microbiol., **75**, 5529-5535
- 10 (2009).
- 11 29. **Takeno, S., Sakuradani, E., Tomi, A., Inohara-Ochiai, M., Kawashima, H., and**
- 12 **Shimizu, S.:** Transformation of oil-producing fungus, *Mortierella alpina* 1S-4,
- 13 using Zeocin, and application to arachidonic acid production, J. Biosci. Bioeng., **100**,
- 14 617-622 (2005).
- 15 30. **Ando, A., Sakuradani, E., Horinaka, K., Ogawa, J., and Shimizu, S.:**
- 16 Transformation of an oleaginous zygomycete *Mortierella alpina* 1S-4 with the
- 17 carboxin resistance gene conferred by mutation of the iron-sulfur subunit of
- 18 succinate dehydrogenase, Curr. Genet., **55**, 349-356 (2009).
- 19 31. **Ito, Y., Muraguchi, H., Seshime, Y., Oita, S., and Yanagi, S. O.:** Flutolanil and
- 20 carboxin resistance in *Coprinus cinereus* conferred by a mutation in the cytochrome
- 21 *b*₅₆₀ subunit of succinate dehydrogenase complex (Complex II), Mol. Genet.
- 22 Genomics, **272**, 328-335 (2004).
- 23 32. **Wynn, J. P. and Ratledge, C.:** Evidence that the rate-limiting step for the
- 24 biosynthesis of arachidonic acid in *Mortierella alpina* is at the level of the 18:3 to

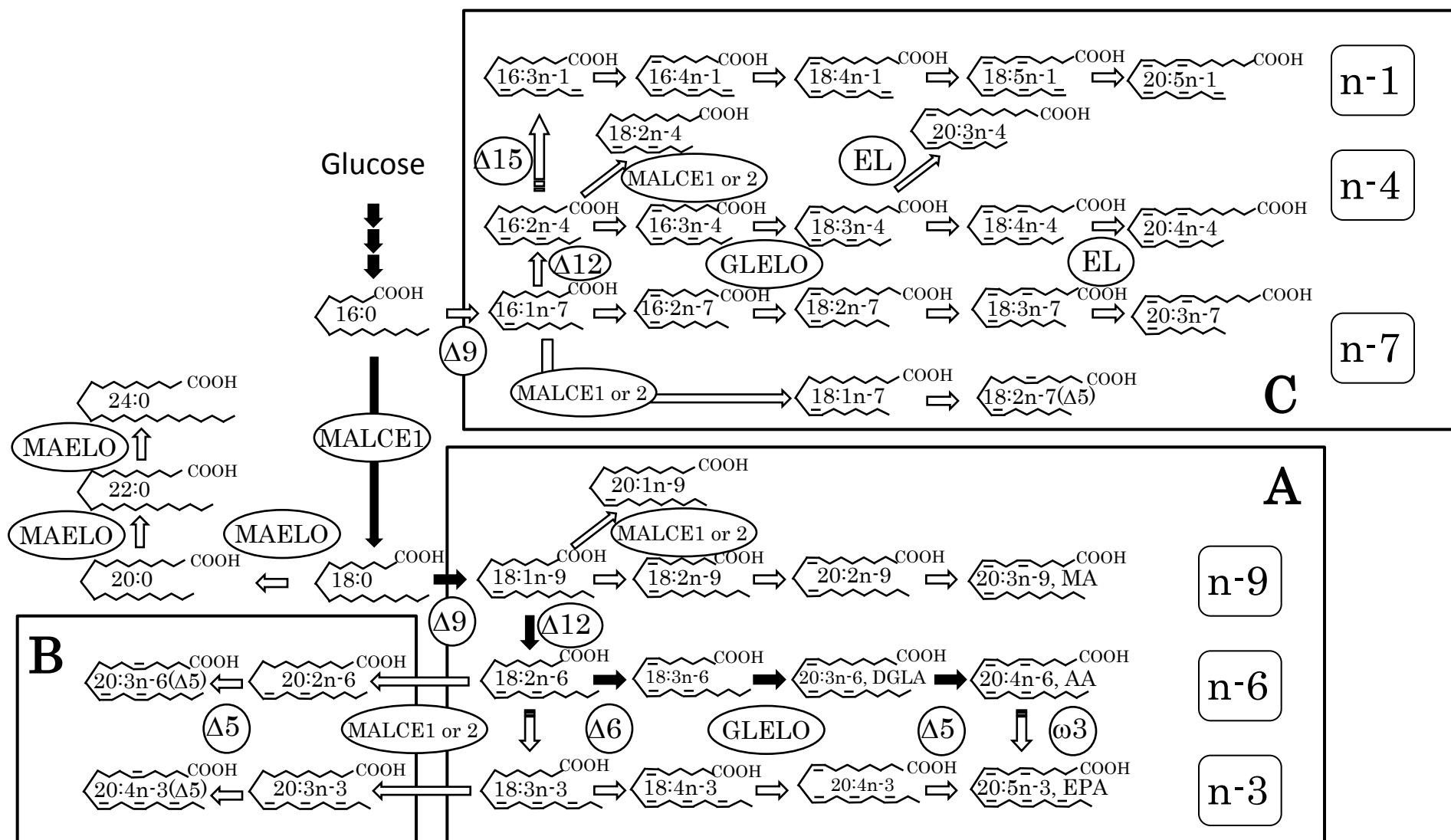
- 1 20:3 elongase, Microbiology, **146**, 2325-2331 (2000).
- 2 33. **Takeno, S., Sakuradani, E., Tomi, A., Inohara-Ochiai, M., Kawashima, H.,**
3 **Ashikari, T., and Shimizu, S.:** Improvement of the fatty acid composition of an
4 oil-producing filamentous fungus, *Mortierella alpina* 1S-4, through RNA
5 interference with Δ 12-desaturase gene expression, Appl. Environ. Microbiol., **71**,
6 5124-5128 (2005).
- 7 34. **Takeno, S., Sakuradani, E., Murata, S., Inohara-Ochiai, M., Kawashima, H.,**
8 **Ashikari, T., and Shimizu, S.:** Molecular evidence that the rate-limiting step for
9 the biosynthesis of arachidonic acid in *Mortierella alpina* is at the level of an
10 elongase, Lipids, **40**, 25-30 (2005).
- 11

Figure legends

FIG. 1. Pathways for the Biosynthesis of PUFAs in *M. alpina* 1S-4 and its Mutants.

The n-3, n-6, and n-9 PUFAs are derived from 18:1n-9 (A), the nonmethylene-interrupted PUFAs are detected in $\Delta 6$ desaturase-defective mutants (B), and the n-1, n-4, and n-7 PUFAs are derived from 16:1n-7 (C). Black arrows show the AA biosynthetic pathway in the parental strain, *M. alpina* 1S-4. AA, arachidonic acid; ΔN , ΔN desaturase; DGLA, dihomo- γ -linolenic acid; EL, fatty acid elongase; EPA, eicosapentaenoic acid; MA, Mead acid; $\omega 3$, $\omega 3$ desaturase.

FIG. 2. Conversion of n-6 PUFAs to n-3 PUFAs by $\omega 3$ desaturase from *M. alpina* 1S-4 (A) and chromatograms of fatty acid methyl esters prepared from fungal cells. The strains were cultivated at 12°C for 11 days.



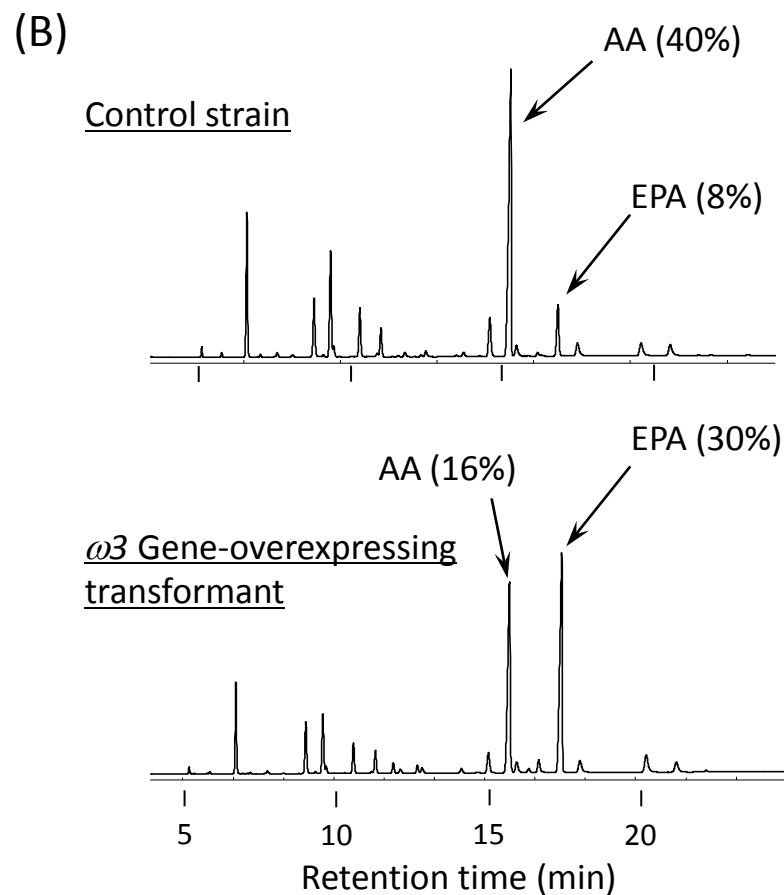
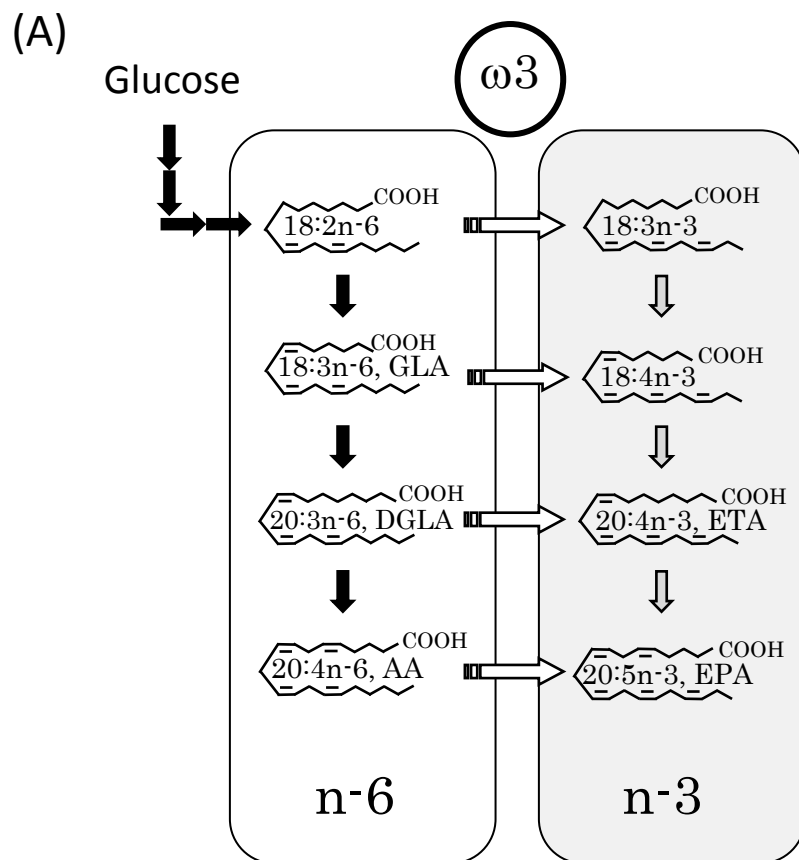


TABLE 1. Lipid productivities of *Mortierella alpina* 1S-4 mutants

Deficient Enzyme	Mutation site	Parent strain	Mutant	Accumulation	Reference
Δ9	G265D	1S-4	T4	18:0 (40%)	13
Δ12	P166L	1S-4, Mut48 ^b , and M209-7 ^b	JT-180	MA (2.6 g /L, 49% of total fatty acids)	14
Δ12 and Δ5	P166L in (Δ12) and W301Stop (Δ5)	1S-4 and Mut48 ^b	M226-9	Enhanced activities of Δ5 and Δ6 desaturases 20:2n-9 (2.2 g/L, 37%)	15
Δ6	Incorrect splicing	1S-4	Mut49	20:3n-6(Δ5) (0.48 g/L, 7%)	16
Δ5	Incorrect splicing	1S-4	S14	DGLA (4.1 g/L, 42%) and AA content (<1%)	17
ω3	W232Stop	1S-4	Y11	AA (1.5 g/L, 45%) without n-3 PUFAs	18
EL1	H154Y and T185I	1S-4	M1	16:0 (30%), 16:1n-7 (8%), and n-4/n-7 PUFAs (30%)	—
N.D. ^a	—	1S-4	KY1	Diacylglycerol (30% of total lipids)	—
N.D.	—	1S-4	V6	Lipid excretion (10-40% of total lipids)	—

^aN.D., not determined.

^bMutants derived from *M. alpina* 1S-4.

TABLE 2. Molecular Breeding of *M. alpina* 1S-4 and Its Mutants for PUFA Production

Accumulated PUFA	Host ^a	Target gene ^b	Method ^c	Note
AA	JT-180	$\Delta 12$	OE	Higher AA production in JT-180 (2.0 g/l/7 days, 39% of total fatty acids) than wild strain 1S-4 (1.2 g/l/7 days, 21%)
AA	1S-4	<i>malce1</i>	OE	Higher accumulation of AA in a transformant (0.76 g/l/6 days, 34%) than wild strain 1S-4 (0.68 g/l/6 days, 28%)
AA	1S-4	<i>glelo</i>	OE	Higher AA production in a transformant (3.6 g/l/10 days, 28%) than wild strain 1S-4 (1.9 g/l/10 days, 19%)
AA	1S-4	<i>PavΔ5</i>	OE	A higher rate of AA (40%) and a lower rate of DGLA (1%) in a transformant than rates of AA (35%) and DGLA (4%) in wild strain 1S-4
AA	1S-4	<i>OstΔ6</i>	OE	A higher rate of AA (44%) in a transformant than (35%) that in wild strain 1S-4
EPA	1S-4	$\omega 3$	OE	High EPA production (0.8 g/l/11 days, 30%)
20:4n-3	S14	$\omega 3$	OE	High 20:4n-3 production (1.8 g/l/11 days, 35%)
22:4n-6, 22:5n-3	1S-4	<i>PavELO</i> , $\omega 3$	OE	Detection of small amounts of 22:4n-6 and 22:5n-3 in wild strain 1S-4
MA	1S-4	$\Delta 12$	Ri	Accumulation of n-9 PUFAs
20:3n-6($\Delta 5$), 20:2n-6	1S-4	$\Delta 6$	Ri	Accumulation of 20:3n-6($\Delta 5$) and 20:2n-6
16:0, 16:1n-7	1S-4	<i>malce1</i>	Ri	Accumulation of 16:0 and 16:1n-7
n-4/n-7 PUFA	M1	<i>maelo</i>	Ri	Accumulation of n-4/n-7 PUFAs and decrease of n-6 PUFAs
n-7 PUFA	M1	$\Delta 12$	Ri	Accumulation of n-7 PUFAs and decrease of n-4 PUFAs
18:0, PUFA	1S-4	<i>maelo</i>	Ri	No accumulation of 22:0 and 24:0, and small increases in 18:0 and the following n-6 PUFAs

^aJT-180, $\Delta 12$ desaturase activity-defective mutant (see TABLE 1); M1, EL1 elongase activity-defective mutant; S14, $\Delta 5$ desaturase activity-defective mutant.

^bThe genes, except for *PavΔ5*, *OstΔ6*, and *PavELO*, were all derived from *M. alpina* 1S-4.

^cOE, overexpression; Ri, RNAi.